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The M Cell as a Portal of Entry to the Lung for the Bacterial Pathogen *Mycobacterium tuberculosis*

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Summary

***M. tuberculosis* accesses the terminal lung and is phagocytosed by alveolar macrophages. Utilizing a mouse intratracheal challenge model, we demonstrate that *M. tuberculosis* rapidly enters through M cells as well. From there, bacilli are deposited within associated intraepithelial leukocytes and subsequently conveyed to the draining lymph nodes early after infection. Osteopetrotic (*Csfm^{op}/Csfm^{op}*) mice, null mutants for macrophage colony-stimulating factor, possess diminished numbers of circulating monocytes and tissue macrophages. *Csfm^{op}/Csfm^{op}* mice were highly susceptible to challenge with *M. tuberculosis*. In contrast to controls, tubercle bacilli were not conveyed to draining lymph nodes early after infection but were instead retained within the mucosa. These results indicate that M cells represent an alternate portal of entry for *M. tuberculosis*, which may contribute to the rapid development of protective lung immune responses.**

Introduction

The alveolar macrophage is generally thought to be the point of entry of *M. tuberculosis* into the lung and the first line of defense against infection by the tubercle bacillus (Henderson et al., 1963). Airborne bacilli are inhaled in droplet nuclei, small droplets that circulate in air for long periods of time, and gain access to the terminal ramifications of the respiratory tree (Wells et al., 1947). However, only particles less than 5 μm in diameter can gain access to the alveolus (Hatch, 1942), where within the alveolar space, resident macrophages phagocytose the tubercle bacillus. Presumably, most bacteria engulfed by alveolar macrophages are removed from the body by the mucocilliary escalator, while a few viable bacilli are thought to be transported in macrophages into the lung. These bacilli within the terminal alveolus represent the source of a primary tuberculous focus or lesion in which the pathogen either replicates or is contained.

Numerous enteric pathogens have been found to utilize a route of entry across the mucosa into the body, namely through M cells. M cells are specialized epithelial cells that transcytose particles across epithelial barriers to an intraepithelial lymphoid pocket created by the modification of the basolateral surface of the M cell (Neutra et al., 1996b). Among pathogens exploiting this pathway to cross the epithelial barrier in the gut are viruses, including reovirus and poliovirus, (Wolf et al., 1981; Sicinski et al., 1990) and bacteria, including *Salmonella*, *Shigella*, *Vibrio*, *Escherichia*, and *Yersinia* species (Inman and Cantey, 1983; Fujimura, 1986; Kohbata et al., 1986; Owen et al., 1986; Wassef et al., 1989; Grutzkau et al., 1990; Fujimura et al., 1992; Jones et al., 1994). A few reports have indicated M cells can be found in the lung as well as in the gut of experimental animals, and previous work has established that particulate antigens (Tenner-Racz et al., 1979) and latex beads (Pappo and Ermak, 1989) can be transported through pulmonary and intestinal M cells. While numerous pathogens have been demonstrated to traverse the gastrointestinal M cell, there is only a single report that a pathogen, reovirus, can use M cells to gain access to the body from the lung (Morin et al., 1994). This suggests that M cells could play a role in luminal sampling of mucosal antigens and pathogens in the lung (Sminia et al., 1989), as well as the gut (Neutra et al., 1996a). M cells possess acidified compartments containing cathepsin E (Finzi et al., 1993) and express MHC class II molecules (Allan et al., 1993) and ICAM-1 on their cell surface (Ueki et al., 1995), shown in some instances to provide costimulatory activity (Damle et al., 1992; Van Seventer et al., 1992; Semnani et al., 1994), and therefore, the machinery necessary for initiating an immune response at the mucosal surface. Professional antigen-presenting cells, macrophages and dendritic cells, have access to the lymphoid pocket (Beinenstock et al., 1973; Gong et al., 1992) and can present antigen to the lymphocytes present in the lymphoid compartment within the mucosa (Beinenstock and Johnston, 1976). While these specialized epithelial cells allow for pathogen entry, they can concomitantly facilitate, and possibly initiate, an early immune response providing protection to the host from the invading pathogen.

Because M cells are known to provide a rapid transit of antigens and pathogens to the immune system, and because the nature of the early immune response to *M. tuberculosis* infection may be determinative in restricting the growth of the pathogen, we have investigated the interaction of virulent tubercle bacilli and M cells following pulmonary challenge in mice. The rationale for such an interaction derives from observations made by Calmette et al. (1933) that children receiving bacille Calmette-Guerin (BCG), the attenuated vaccine strain derived from *M. bovis*, orally, had detectable bacteremia within 3–5 hr (Calmette et al., 1933), indicating rapid transit across the gut epithelium. Subsequently, BCG was observed to cross the gastrointestinal mucosa

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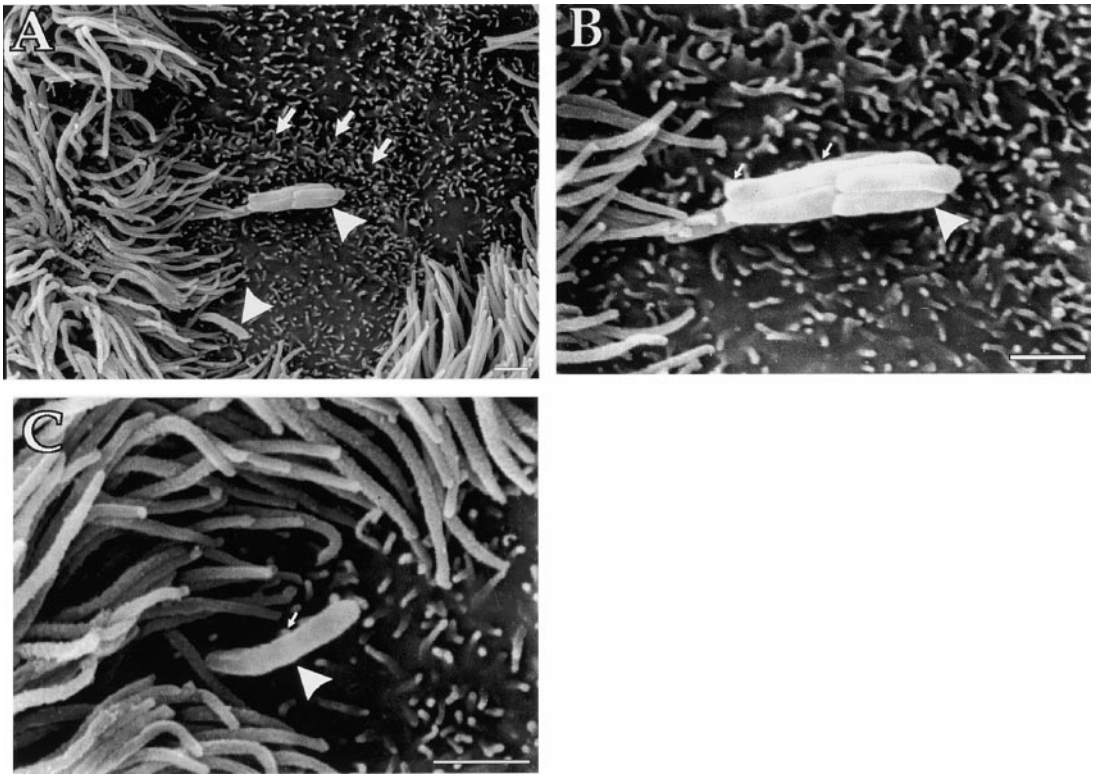

Figure 1. Interaction of *M. tuberculosis* with the Surface of Pulmonary M Cells

C57BL/6 mice were challenged with 1×10^7 virulent *M. tuberculosis*, Erdman strain, intratracheally. Tissue was processed for scanning electron microscopy.

(A) *M. tuberculosis* (arrowheads) adhere to and cause a tunneling of the M cell surface (arrows). Bar, 1 μm; magnification, 2,500×.

(B) Higher magnification of bacteria in (A), with cluster of *M. tuberculosis* (arrowhead) adhering to microvilli on the M cell surface (small arrows). Bar, 1 μm; magnification, 10,000×.

(C) Higher magnification of bacillus in (A), showing microvilli adherence to bacterial surface as well (arrows). Bar, 1 μm; magnification, 13,000×.

through M cells in a rabbit ileal loop model (Fujimura, 1986). Tenner-Racz et al. (1977) have demonstrated that BCG upregulates the number of M cells in the respiratory mucosa in rabbits intratracheally challenged with the bacilli, facilitating soluble antigen transport across the epithelial barrier of the respiratory mucosa.

In the present studies, we address the question of whether *M. tuberculosis* utilizes the M cell as a portal of entry into the body, thus providing a significant conduit for transit of the pathogen to the draining lymph nodes. Utilizing both conventional mice and osteopetrotic (*Csfm*^{op}/*Csfm*^{op}) mice homozygous for a mutation in the mononuclear phagocytic growth factor, colony-stimulating factor 1 (Pollard and Stanley, 1996), we present evidence that pulmonary M cells represent a novel alternate entry site for *M. tuberculosis* and suggest that this could contribute to the development of an early protective response.

Results

M. tuberculosis Adheres to the Surface of M Cells in the Bronchial Mucosa 1 hr Post Respiratory Infection

To investigate whether *M. tuberculosis* interacts with cells lining the respiratory mucosa, and if so, to establish the kinetics of that interaction, BALB/c and C57BL/6 mice, free of known infectious pathogens, were infected intratracheally and intranasally with virulent *M. tuberculosis*. Mucosal tissue lining the respiratory tree was sampled, beginning from areas just distal to the first bronchial bifurcation of the airways. Adherence of *M. tuberculosis* to the mucosal surface was evaluated utilizing scanning electron microscopy (SEM). *M. tuberculosis* (Figure 1A, arrowheads) intimately contacted the apical surface of M cells and initiated entry. The microvilli of the M cells adhered to the surface of the bacterial cluster (Figure 1B, small arrows), and a tunneling of the cell surface was seen (Figure 1A, large arrows). In Figure 1A (arrowhead), an additional single bacillus can be seen adhering to the cell surface, and contact with the microvilli is evident at higher magnification as well (Figure 1C, small arrows). In the six infected mice subjected to detailed evaluation, adherence of *M. tuberculosis* to M cells was found at various points along the mucosa from the first bronchial bifurcation, extending as far as to the bronchioles. Control mice intratracheally injected with PBS supplemented with Tween detergent showed no bacilli bound to the epithelial surface, independently confirming that these mice were free of respiratory pathogens. By 3 hr post infection fewer bacilli could be found adhering to the M cell surface, indicating that many of the bacilli had been internalized or removed. Although periodically bacilli could be seen in contact

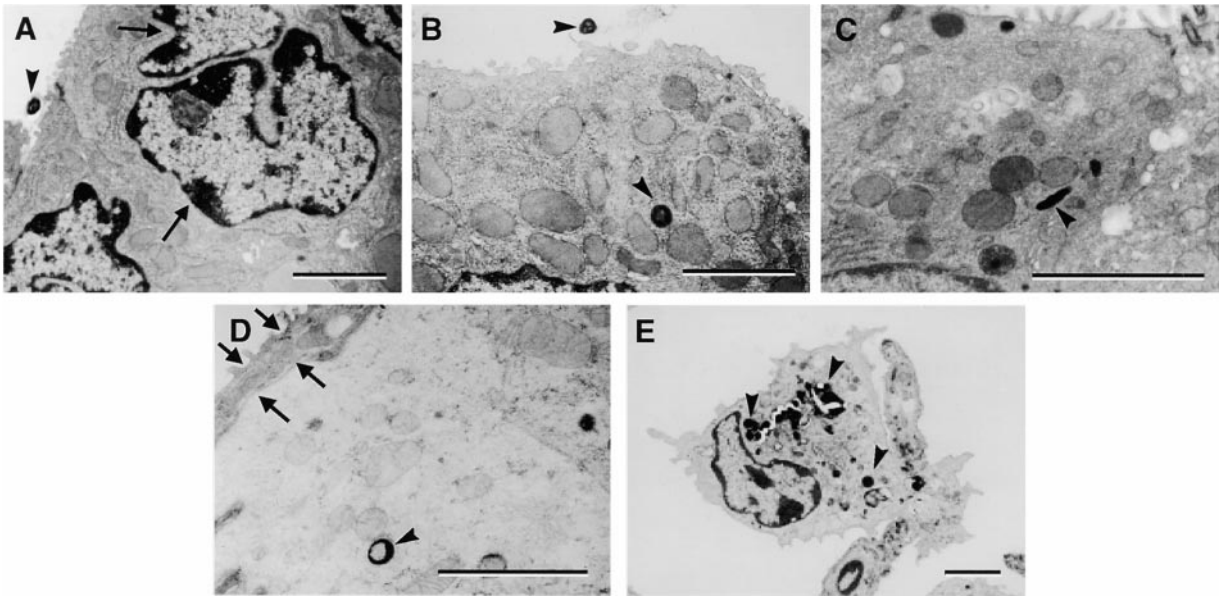

Figure 2. *M. tuberculosis* Internalization within Pulmonary M Cells and Intraepithelial Leukocytes

(A) *M. tuberculosis* (arrowhead) can be seen adhering to the M cell surface at a region of lymphoid cell accumulation (arrows), by 3 hr post infection. Magnification, 5000×.

(B) *M. tuberculosis* (arrowheads) binds to the M cell surface and is internalized. *M. tuberculosis* can be seen within M cells in cross section. Magnification, 6000×.

(C) *M. tuberculosis* is internalized within M cells seen here in longitudinal section (arrowhead). Magnification, 9500×.

(D) Ultimately, *M. tuberculosis* (arrowhead) is transcytosed across the M cell surface (smaller arrows) and is found within intraepithelial macrophages (larger arrows denote cell border) Magnification, 8000×.

(E) Numerous bacilli (arrowheads) can be found within alveolar macrophages at this time. Magnification, 5000×; bar, 2.5 μm.

with ciliated epithelial cell surfaces, *M. tuberculosis* could never be discerned interacting with the plasma membranes of these cells, nor were there any observable modifications of the cell surface. Thus, SEM established a unique interaction between specialized epithelial cells and *M. tuberculosis*. To establish a role for M cells in facilitating *M. tuberculosis* entry across the respiratory mucosa, however, requires a more detailed examination by transmission electron microscopy (TEM).

M. tuberculosis Can Be Found within M Cells by 3 hr Post Infection

While *M. tuberculosis* was observed binding to the M cell surface as early as 1 hr post respiratory infection by SEM, the presence of the tubercle bacilli within M cells could be demonstrated by TEM by 3 hr post infection (Figure 2B, arrowhead). In the examples presented, the bacilli are seen in both cross section (Figure 2B) and longitudinal section (Figure 2C, arrowhead) within M cells. The basolateral modification of the murine lung M cell surface is not seen as readily in these sections, in contrast to other rodent models such as the rat or rabbit, because mice generally have less organized pulmonary lymphoid tissue (Gregson et al., 1979; Sminia et al., 1989). Despite the lack of discrete lymphoid organization within the mucosa of these mice, lymphoid cells can clearly be discerned at regions of *M. tuberculosis* uptake within the mucosa (Figure 2A, arrows), as well as in the submucosa, indicating that this is a region of bronchus-associated lymphoid tissue (BALT) localization. Furthermore, in Figure 2D, *M. tuberculosis* (arrowhead) was seen to be transcytosed across the M cell surface (small arrows) and delivered to macrophages within the lymphoid pocket (larger arrows designate macrophage border).

More than 30 mice were evaluated by TEM in tissue harvested at numerous times post infection, and sections were taken at each time from many locations along the respiratory mucosa. In mice challenged with *M. tuberculosis* by the intratracheal route, bacterial presence within the M cells was not common and found only after detailed examination. However, intranasal infection yielded greater numbers of infected M cells. *M. tuberculosis* could readily be seen within alveolar macrophages as early as 1 hr post infection, and multiple bacilli were seen by 3 hr post infection (Figure 2E, arrowheads). In contrast, although *M. tuberculosis* binding to M cells was seen by 1 hr post infection, no bacilli were seen within M cells by TEM until 3 hr post infection. While these experiments clearly establish that *M. tuberculosis* utilizes the M cell pathway as a means of host invasion, the frequency of entry within M cells is much less than that in the alveolar macrophages, and the kinetics of entry differ as well.

M. tuberculosis Crossing the Respiratory Epithelia or Entering the Lung Parenchyma Access Different Lymph Nodes, with Differing Kinetics

To evaluate the possible contribution of bacterial entry across the mucosal barrier toward an early immune response, lymph nodes draining the respiratory tree were dissected, homogenized, and plated for colony-forming

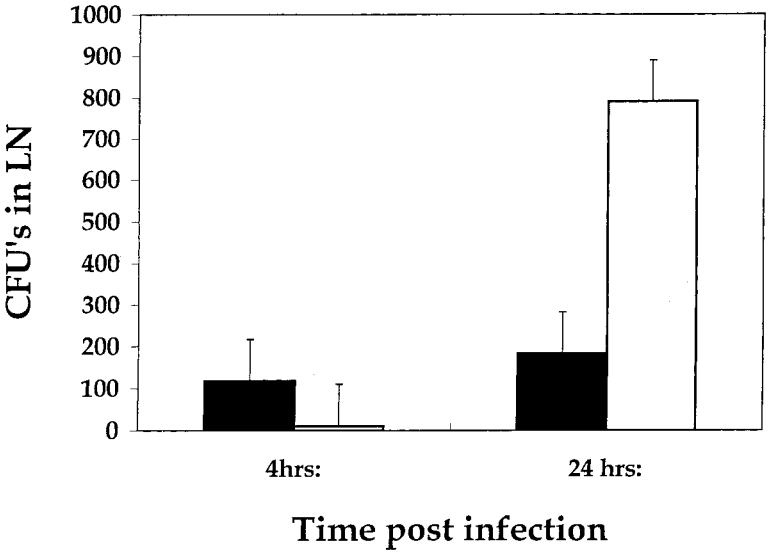

Figure 3. Differential Accumulation of Mycobacteria from the Mucosa and the Terminal Alveolus within Their Respective Draining Lymph Nodes

Mycobacteria accessing the lymphatic system from the mucosa accumulate more readily in the draining lymph nodes than do bacilli entering the lymphatic system from the terminal alveolus. Normal C57BL/6 mice were infected intranasally with 1×10^7 BCG, and BTLN (filled bars) and PBLN (open bars) were differentially plated for cfu 4 and 24 hr post infection. Data presented as the mean ± SD from two mice harvested in each of three independent experiments.

units (cfu). Enumeration of viable bacilli within the bronchotracheal lymph nodes (BTLN) as well as the pulmonary and bronchopulmonary lymph nodes (PBLN) was carried out at early and later times post infection. Our prediction was that there would be differing kinetics of bacterial deposition within the nodes as a consequence of the bacterial point of entry. In mice assayed in three independent experiments, as early as 4 hr post intratracheal infection, numerous cfu could be found within the BTLN (black bars) (Figure 3). PBLN (white bars) plated from the same mice give negligible cfu at 4 hr post infection. By 24 hr post infection, while the number of bacilli doubled in the BTLN, the number of bacilli in the PBLN had now risen by 100-fold. Although both intraepithelial and alveolar macrophages contain mycobacteria by 3 hr, as was demonstrated by the TEM studies above, only macrophages arising from the intraepithelial pocket convey bacilli at 4 hr post infection to the BTLN lymph nodes. Alveolar macrophages maintain their mycobacteria in the alveolus, and bacteria do not gain access to the PBLN until some time later. These results suggest that tubercle bacilli gaining access to the epithelial lymphoid tissue through M cell transcytosis are likely to initiate immune responses earlier than those found in the alveolar macrophages.

Osteopetrotic Mice Are Highly Susceptible to *M. tuberculosis* Infection

To investigate whether the paucity of demonstrable bacilli within the M cells seen by TEM was due to extremely rapid bacterial transcytosis, the small tissue sampling area assessed microscopically, a low frequency of bacterial entry, and to determine the existence of a physiological significance for this entry route, mice homozygous for the osteopetrotic mutation (*Csfm^{op}/Csfm^{op}*) provided a useful model. *Csfm^{op}/Csfm^{op}* mice have a null mutation in the gene encoding macrophage colony-stimulating factor (M-CSF), also known as CSF-1, resulting in a lack of production of the growth factor (Pollard and Stanley, 1996). As a result, these mice have few circulating monocytes and fewer resident macrophages, depending upon the organ examined (Cecchini et al., 1994). Should transcytosis of bacilli across M cells be significant for host survival, we hypothesized that mice impaired in their ability to recruit monocytes to the mucosa would be more susceptible to *M. tuberculosis* infection. To test whether these mice are impaired in their ability to control *M. tuberculosis* infection, we infected *Csfm^{op}/Csfm^{op}* and their homozygous wild-type littermates with 1×10^3 *M. tuberculosis*. In two independent experiments, while control mice survive over 10 weeks, *Csfm^{op}/Csfm^{op}* mice succumbed by 4 weeks post infection (Figure 4A). Plating of the lung homogenate obtained at the time of death from *Csfm^{op}/Csfm^{op}* mice ironically yielded similar cfu as did the C57BL/6 strain (Figure 4B), despite the enhanced mortality of *Csfm^{op}/Csfm^{op}* mice to *M. tuberculosis* infection. Thus, the alveolar macrophages in *Csfm^{op}/Csfm^{op}* mice were able to control *M. tuberculosis* replication adequately.

The only known mechanism of direct macrophage mycobactericidal activity is through the production of reactive nitrogen intermediates (Chan et al., 1992). Since the number of recoverable cfu from *Csfm^{op}/Csfm^{op}* mice at end-stage disease was comparable to controls, we sought to determine whether this killing mechanism was intact in *Csfm^{op}/Csfm^{op}* mice. Lung tissue from infected control and *Csfm^{op}/Csfm^{op}* mice was harvested at end-stage disease, and immunohistochemistry was performed to determine the presence of the iNOS protein (Figures 4C and 4D). Both control and *Csfm^{op}/Csfm^{op}* mice produced the iNOS protein, in comparable amounts. Taken together these results indicate that alveolar macrophages in *Csfm^{op}/Csfm^{op}* mice are not impaired in their ability to control *M. tuberculosis* infection, and thus are unlikely to be responsible for the enhanced susceptibility of these mice.

Csfm^{op}/Csfm^{op} Mice Reveal Prolonged Retention of *M. tuberculosis* within Respiratory Mucosa

To address the role of monocyte recruitment to mucosal surfaces in conveying bacilli to the draining lymph nodes, mucosal tissue from *Csfm^{op}/Csfm^{op}* and control mice was processed for light microscopy at or near lymphatic vessels, and the presence of acid-fast bacilli

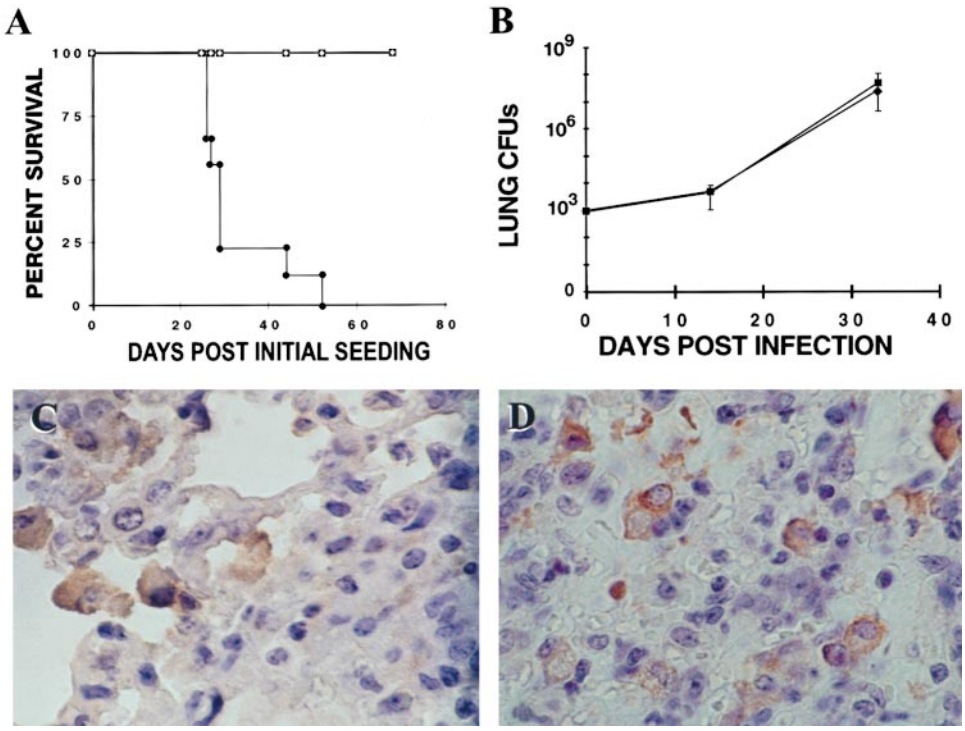

Figure 4. Mortality and Lung Bacterial Burden in *Csfm^{op}/Csfm^{op}* Mice Lacking CSF-1

(A) Survival of *Csfm^{op}/Csfm^{op}* mice (filled circle) (9 mice/group), wild-type controls (filled square) (5 mice/group), and C57BL/6 mice (open diamond) (3 mice/group) challenged intratracheally with 10^3 virulent *M. tuberculosis*. (Data are the combination of two separate experiments of *Csfm^{op}/Csfm^{op}* versus each control.)

(B) Number of cfu recovered from the lungs of *Csfm/Csfm^{op}* (filled square) and C57BL/6 (filled diamond) mice challenged with 10^3 *M. tuberculosis* (3 mice harvested per group per time point).

(C) *Csfm^{op}/Csfm^{op}* mice produce iNOS in response to *M. tuberculosis* challenge. Immunohistochemistry of lung tissue from *Csfm^{op}/Csfm^{op}* mice challenged with 1×10^3 *M. tuberculosis* IT reveals iNOS localized within lung macrophages at 3 weeks post infection ($n = 3$).

(D) Immunohistochemistry for the detection of iNOS in similarly challenged C57Bl/6 mice reveals comparable iNOS production.

(AFB) was examined. Maintaining *M. tuberculosis* within the epithelia in the *Csfm^{op}/Csfm^{op}* mouse may reflect a paucity of macrophages in the *Csfm^{op}/Csfm^{op}* mice, a macrophage migration defect, or a longer retention time of the bacilli within the M cell. *M. tuberculosis* could be detected in the epithelia in *Csfm^{op}/Csfm^{op}* mice as early as 3 hr post infection (Figure 5A) and as late as 3 days post infection (Figure 5B). *M. tuberculosis* (arrowheads) was readily found in the bronchiolar epithelium of *Csfm^{op}/Csfm^{op}* mice without any accompanying lymphoid cells within the epithelia. *M. tuberculosis* could not be readily discerned within the epithelia of control mice at any time point; however, previous demonstration by TEM indicated that *M. tuberculosis* residence within M cells was usually in association with intraepithelial leukocytes. In contrast, *M. tuberculosis* (arrowheads) was seen within the draining lymphatic vessel (arrow) by 24 hr post infection in C57BL/6 mice (Figures 5C and 5D). By 3 days post infection, tubercle bacilli (arrowhead) were still impaired in their ability to access nearby lymphatic vessels in the *Csfm^{op}/Csfm^{op}* homozygous null mutant mice (Figure 5B, arrow). We infer that it is the relative absence of the lymphoid cells, particularly macrophages, early in infection in the *Csfm^{op}/Csfm^{op}* mucosa that results in the persistence of the tubercle bacilli within the M cells. The absence of migratory cells in the *Csfm^{op}/Csfm^{op}* mucosa able to convey the bacilli to the draining lymphatic vessels enables detection of the bacilli accumulating within the epithelia.

To further demonstrate a quantitative difference in the number of bacilli persisting within the *Csfm^{op}/Csfm^{op}* mucosal tissue early after infection, we microdissected *M. tuberculosis*-infected mucosal tissue away from lung parenchyma and plated for the number of recoverable cfu. At 36 hr post infection, 10-fold more bacilli were recovered from *Csfm^{op}/Csfm^{op}* mucosal tissue, as compared to controls (Figure 6A) ($p \leq 0.0001$, Student's t test). These findings confirm that bacilli persist longer within *Csfm^{op}/Csfm^{op}* mucosa, early after infection, probably as a result of a failure to convey bacilli to the draining lymphatics.

Csfm^{op}/Csfm^{op} Mice Are Impaired in Their Ability to Convey Bacilli to the Draining Lymph Nodes

In order to verify that bacterial retention within the mucosa results in a failure of the bacilli to access the draining lymph nodes in *Csfm^{op}/Csfm^{op}* mice, we harvested lymph nodes 36 hr post infection to determine their bacterial burden (Figure 6B). While C57BL/6 mice have detectable cfu within both BTLN and PBLN, *Csfm^{op}/Csfm^{op}* mice do not. Thus, early after infection, bacilli

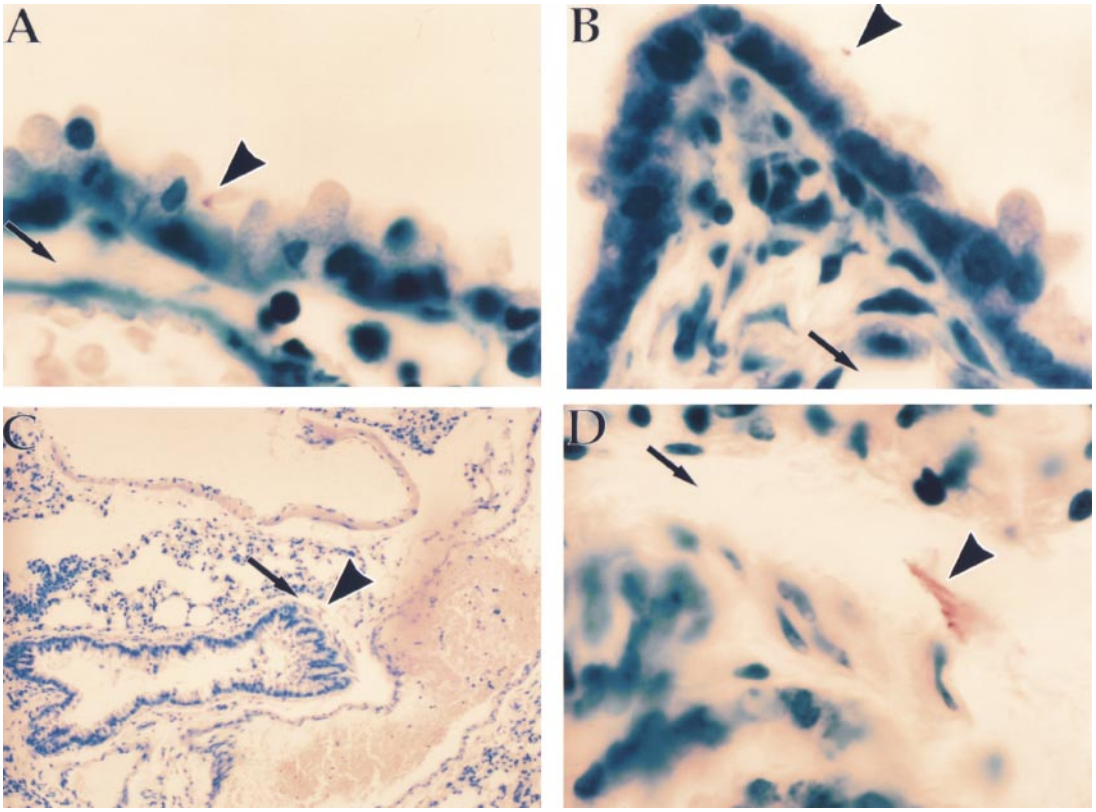

Figure 5. Retention of *M. tuberculosis* within the Intraepithelial Leukocytes of *Csfm*^{op}/*Csfm*^{op} Mice 3 Weeks Post Infection

Bronchiolar tissue was found to contain acid-fast bacilli (arrowheads) within the epithelia at 3 hr (A) and 3 days (B) post infection in *Csfm*^{op}/*Csfm*^{op} mice (magnification, 600×; bar, 25 μm). In contrast, in the C57BL/6 controls, *M. tuberculosis* (arrowhead) can be seen within lymph vessels (arrow) draining the bronchiole by 24 hr post infection (C) (magnification, 165×). Higher magnification (D) demonstrates several acid-fast bacilli (arrowhead) within the vessel (arrow) (magnification, 600×; bar, 25 μm).

are retained within the mucosa and therefore prevented from accessing the lymphatic system.

Discussion

One of the fundamental questions in tuberculosis concerns determinants of progression to disease following exposure and infection. The factors responsible for the development of complete resistance or latent infection in the majority of immunocompetent hosts relative to those who develop primary progressive or reactivated forms of disease remain unclear. The early development of an appropriate immune response has an important determinative role on the outcome of infection. In studies using genetically resistant and susceptible rabbits, for example, Lurie et al. (1955) established that a greater percentage of the tubercle bacilli instilled were routed earlier to the draining lymph nodes in resistant rabbits, in response to aerosol challenge, correlating with their enhanced survival. In other studies on naive guinea pigs, Riley et al. (1959) demonstrated that infectious particles carried in room air from patients in a tuberculous ward inhaled by naive guinea pigs resulted in PPD conversion of the guinea pigs, yet did not always produce a primary lesion. Some of these cases resulted in cultivable bacilli from the hilar lymph nodes, indicating that despite the inhalation of a similar number of infectious particles as controls, systemic sensitization and PPD conversion could occur in the absence of primary lesion formation, precluding disease development. Obvious possible interpretations are that the mucocilliary escalator cleared a greater number of bacilli in some animals, which were then unable to reach the terminal ramifications of the respiratory tree, or that the phagocytic activity of the host macrophages determined the number of bacilli able to form lesions (Henderson et al., 1963). Yet these hypotheses do not address the PPD conversion of the host, an indication that some bacilli were maintained for a long enough period of time to facilitate immune system recognition. An additional hypothesis suggested by the present work would be that some of these bacilli entered the lung at an alternate site and were rapidly conveyed to the lymph nodes, where they engendered a protective cell-mediated immune response. This scenario would prevent primary parenchymal lesion development, yet still result in systemic PPD reactivity. Paradoxically, additional reports of aerogenically challenged guinea pigs have demonstrated that infection with minimal numbers of bacilli do not yield cultivable bacilli in the hilar nodes until at least 10 days post infection (Smith et al., 1970; McMurray, 1994). This discrepancy may be attributable to differences in infection protocol, as well as innoculum size. Clearly the reconciliation of these findings will give rise to a better understanding of bacterial trafficking to the draining lymph nodes.

The present work was undertaken to explore whether

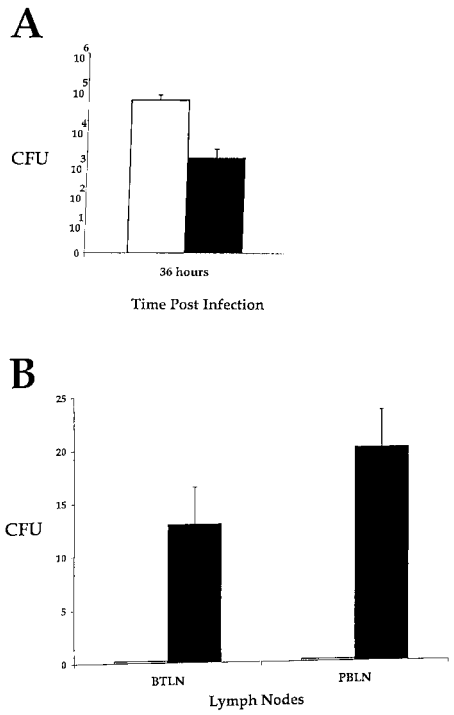

Figure 6. *Csfm*^{op}/*Csfm*^{op} Mice Retain Bacilli within the Mucosa Early after Infection, Preventing Deposition within the Draining Lymph Nodes

(A) *M. tuberculosis* is retained within the *Csfm*^{op}/*Csfm*^{op} mucosa, as compared to controls, early after infection. C57Bl/6 mice (black bars) and *Csfm*^{op}/*Csfm*^{op} mice (white bars) were challenged with 1×10^7 *M. tuberculosis* IN. At 36 hr post infection, lungs and airway were removed, and mucosal tissue was microdissected away from lung parenchyma, homogenized, and plated for cfu. Data are pools of tissue from three animals per group, presented as mean $\pm$ SD, and were evaluated by the Student's t test ($p \leq 0.0001$).

(B) *M. tuberculosis* fails to access the draining lymph nodes 36 hr post infection, at a time when they are retained within the mucosa. BTLN and PBLN from C57Bl/6 mice (black bars) and *Csfm*^{op}/*Csfm*^{op} mice (white bars) were harvested 36 hr post infection and plated for cfu ($n = 3$) ($p \leq 0.001$).

M cells existing in the gut and lung could serve as a portal of entry for *M. tuberculosis* in addition to alveolar macrophages. M cells facilitate rapid transcytosis of microbial pathogens across the mucosa and into organized lymphoid tissue. Presumably, this expedites the initiation and development of rapid immune responses locally, perhaps with M cells presenting antigen to T cells within the epithelial pocket (Sminia et al., 1989). A second question has been whether pulmonary M cells accelerate the transit of *M. tuberculosis* within macrophages to the draining lymph nodes, perhaps facilitating bacterial dissemination as well as initiating rapid immune responses. M cell transcytosis has been shown here to facilitate *M. tuberculosis* conveyance to the BTLN as early as 4 hr post infection. Our data therefore suggest the early development of local responses in the lung.

Although *M. tuberculosis* could be readily seen within alveolar macrophages as early as 1 hr post infection, transit through M cells is difficult to visualize using TEM, presumably in part because of the rapidity of transcytosis. In this context, the osteopetrotic null mutant mouse served as a useful model to address questions of mycobacterial transit. These mice lacking CSF-1 have a major deficit in the number of peripheral blood circulating monocytes (Wiktor-Jedrzejczak et al., 1982, 1992) and hence are limited in the number of macrophages that can be recruited from the blood into the intraepithelial compartment. If there was a deficit in intraepithelial macrophages, we reasoned that *M. tuberculosis* might persist for longer times within M cells. This was confirmed by our experiments in the *Csfm*^{op}/*Csfm*^{op} mouse. Although *M. tuberculosis* enters the M cell within the same time frame as in control mice, *M. tuberculosis* persisted within the epithelial lining as late as 36 hr post infection. Our interpretation is that the "handing off" of bacilli to neighboring macrophages does not rapidly occur in the *Csfm*^{op}/*Csfm*^{op} mice, and hence bacteria persist and accumulate in M cells of *Csfm*^{op}/*Csfm*^{op} mice.

One immunologic consequence of the transcytosis of *M. tuberculosis* through pulmonary M cells would be rapid draining into the bronchotracheal lymph nodes. Lymphatic drainage of the lung is compartmentalized, in that materials deposited in the terminal alveolar sac would be conveyed by pulmonary macrophages to the draining lymph nodes, specifically the pulmonary or bronchopulmonary nodes (PBLN) (Nagaishi, 1972; Netter, 1992). In contrast, material deposited higher up in the respiratory tree, gaining access to the lymphatic vessels in the submucosa, would necessarily first access the bronchotracheal nodes, and secondarily the bronchopulmonary nodes, if the deposition is at a more distal site along the respiratory mucosa (Nagaishi, 1972; Netter, 1992). Thus, inhaled *M. tuberculosis* or other bacilli phagocytosed by an alveolar macrophage would first traffic to the PBLN, and subsequently to the BTLN. Bacilli that had been transcytosed across bronchial M cells and gained access to the nearby lymphatic vessels would, in contrast, be deposited initially within the BTLN. We reasoned that bacilli transcytosing the epithelium through M cells and accessing lymph vessels from the mucosal surface would reach the BTLN earlier than bacilli entering via the alveolar sac, which would seed the PBLN. Our expectation was that mucosally derived transit to the lymph nodes would occur more rapidly than the transit of bacilli within the alveolar macrophages into the pulmonary lymph nodes, as many of these macrophages are removed via the mucociliary escalator and therefore cannot enter the lymphatic system. Our data indicated that *M. tuberculosis* could be cultured from the BTLN at an earlier time than from the pulmonary lymph nodes. In the first 24 hr of infection, we estimate that roughly 10% of the bacilli entering the lymphoid system derive from transcytosis across the epithelial mucosa via the M cell. Thus, a local cellular immune response could be very rapidly initiated that could contribute to localization of primary infections within the parenchyma of the alveolus and possibly restrict bacterial growth and dissemination. Consistent with these observations in conventional mice was the finding of the accumulation of *M. tuberculosis* within M cells much later in infection in the *Csfm*^{op}/*Csfm*^{op} mice, and particularly a diminished number of cfu recovered from the BTLN nodes of the *Csfm*^{op}/*Csfm*^{op} mice, in comparison to challenged control mice. The *Csfm*^{op}/*Csfm*^{op} mice showed increased mortality to pulmonary *M. tuberculosis* challenge as well. Clearly the diminution in numbers

of alveolar macrophages that can serve as effector cells in these mice lacking CSF-1 (Witmer-Pack et al., 1993) contributed to the *Csfm^{op}*/*Csfm^{op}* mouse's enhanced susceptibility. In addition, we would suggest that the inability to readily deliver *M. tuberculosis* to macrophages transiting to the BTLN nodes, where they can initiate a rapid cellular immune response, may also contribute to the greatly increased mortality of the *Csfm^{op}*/*Csfm^{op}* mouse. The cause of death of these mice appeared to be a large accumulation of intraalveolar fluid in addition to extensive tissue necrosis, reminiscent of tuberculous pneumonia seen in immunocompromised patients. Our interpretation is that this enhanced mortality is primarily attributable to an early failure of the immune response, and perhaps secondarily to a paucity of effector cells to combat the infection. Our inference derives from the fact that the mice are challenged with 5×10^3 bacilli, but have roughly 1×10^6 alveolar macrophages (Wiktor-Jedrzejczak et al., 1992), enough to deal with the innoculum. Furthermore, the alveolar macrophages of the *Csfm^{op}*/*Csfm^{op}* mice were fully capable of producing the only known macrophage mycobactericidal product, NO. It is thus unlikely that the sole cause of their enhanced susceptibility is due to diminished numbers or function of alveolar macrophages.

The present experimental evidence suggests M cells in the mouse lung can serve as an additional portal of entry for *M. tuberculosis*, one that allows rapid transit of the organism within macrophages to the draining lymph nodes, which could contribute to the early development of a protective immune response. The CSF-1 deficiency in the *Csfm^{op}*/*Csfm^{op}* mice results in a reduction in the number of macrophages recruited from peripheral blood. This in turn leads to the accumulation of tubercle bacilli in the M cells, a diminution in the number reaching the draining lymph nodes, and ultimately a severe immunocompromise we believe may be responsible for the increased mortality in these animals. The potential of M cells of the lung to deliver bacterial antigens rapidly to the draining lymph nodes suggests that they may be critical to the development of protective immune responses to infection.

Experimental Procedures

Animal Strains

Normal female BALB/C and C57BL/6 were purchased from Jackson Laboratories and were housed under specific pathogen-free conditions in the biosafety level 3 (BSL3) facility at the Albert Einstein College of Medicine, as described previously (Flynn et al., 1995). Mice were 6–8 weeks old and weighed 16–22 g. Male and female osteopetrotic (*Csfm^{op}*/*Csfm^{op}*) mice were bred at AECOM, and since they are toothless, were fed powdered chow (Purina) and 10 cc of enfamil daily, as previously described (Pollard et al., 1991). The mutation is carried on a mixed background of intercrosses between C57BL/6 × C3H mice heterozygous for the *Csfm^{op}*/*Csfm^{op}* phenotype, as described previously (Wiktor-Jedrzejczak et al., 1990). Nullizygous mice were identified by their absence of incisors. Homozygous wild-type (+/+) and C57BL/6 mice were utilized as controls. All mice were housed under specific pathogen-free conditions.

Bacterial Strains

Virulent *M. tuberculosis* Erdman strain was obtained as previously described (Flynn et al., 1995). Briefly, frozen stock solutions of known bacterial titer maintained at −70°C were utilized. Stocks were derived from cultures that had been previously passaged through animals, in order to maintain virulence. BCG (Pasteur) was similarly grown, and stock vials of known bacterial titer were prepared.

Mouse Infection

All experimental procedures conducted were within NIH recommended guidelines, with every attempt made to minimize pain and suffering to subjects. All mouse strains utilized were anesthetized with approximately 65 mg/kg weight of a solution of sodium pentobarbital (Nembutal) diluted in phosphate-buffered saline (PBS) (Sigma). Infections were carried out in a BSL3 containment facility, within a biosafety cabinet. Prior to infection, aliquots were thawed, diluted in PBS supplemented with 0.05% Tween 80, and sonicated for 10 s in a cup-horn sonicator. Mice were infected intratracheally (IT) following their immobilization on a Styrofoam board and surgical exposure of the trachea. A 50 μl suspension of virulent *M. tuberculosis*, or BCG, at a concentration of 1×10^7 in BALB/c and C57BL/6 mice, or 1×10^3 in *Csfm^{op}*/*Csfm^{op}* mice, and their controls, was injected into the trachea. The injection was followed by a puff of air to aid mouse respiration. To reduce the number of anesthesia-related deaths, mice were kept warm postoperatively under a heating lamp within the biosafety cabinet until their awakening. Intranasal (IN) infection with 1×10^7 organisms of either *M. tuberculosis* or BCG was accomplished in similarly anesthetized normal BALB/C and C57BL/6 mice and *Csfm^{op}*/*Csfm^{op}* mice. Twenty-five microliters of the bacterial suspensions was placed into either nostril of the anesthetized mouse, then allowed to recover under the heating lamp as well. The infecting dose was verified by plating the homogenate at 24 hr post infection. At least 30 normal mice infected IT and IN with *M. tuberculosis*, 10 *Csfm^{op}*/*Csfm^{op}* mice infected IT, 10 C57BL/6 mice, and 4 homozygous wild type littermates similarly infected were evaluated in these studies. Twenty BALB/c mice were utilized for IN infections with BCG, six C57BL/6 and six *Csfm^{op}*/*Csfm^{op}* mice were given IN *M. tuberculosis* infections, and two normal BALB/C and C57BL/6 mice were given PBS-Tween intratracheally and intranasally to serve as controls.

Assessment of Disease Progression

Animals were sacrificed by overdose on sodium pentobarbital (Nembutal) at days 1, 7, 14, 21, and 28 after infection, in the *Csfm^{op}*/*Csfm^{op}* mice. Mouse susceptibility to *M. tuberculosis* infection in the various experimental groups was assessed by determination of the time of death; quantitation of viable cfu in livers, lungs, spleens; and by histopathological examination of these organs (Flynn et al., 1995). In order to minimize suffering, mice were sacrificed when moribund and scored as succumbing to *M. tuberculosis* infection.

Quantification of Viable Bacilli in Infected Tissues

Spleen, liver, and lung tissues harvested at 24 hr, 2 weeks, and 4 weeks post infection were asceptically removed from *Csfm^{op}*/*Csfm^{op}* mice, homozygous wild-type control mice, and normal C57BL/6 mice and suspended in PBS solution supplemented with 0.05% Tween 80. Tissues were then homogenized in plastic bags using a Stomacher tissue homogenizer (Tekmar Company, Cincinnati, OH), diluted and plated onto supplemented 7H10 agar (Difco Laboratories, Incorporated, Detroit, MI) to determine the number of viable organisms per organ per time point. Because each organ was partitioned for various studies, tissue portions were harvested for a particular study based on their anatomical location. For cfu quantitation in the lungs, the right lobes of the lung were utilized, in the liver the right lobes were used, and in the spleen the caudate half was used. Colonies were counted after 21 days incubation at 37°C.

Histopathology

Normal C57BL/6 mouse lung tissue was harvested at 1, 3, 4, 24, 48, and 72 hr, and 1, 2, and 4 weeks post infection and were immersion fixed in 10% buffered formalin before embedding in paraffin. *Csfm^{op}*/*Csfm^{op}* mouse tissue was harvested at 1, 3, 24, 48, and 72 hr, and 1, 2, and 4 weeks post infection, as were their heterozygous control mouse tissues. Homozygous wild-type control mouse tissue was harvested at 24 hr, 2 weeks, and 4 weeks post infection, and similarly harvested and embedded. Thick sections (4 μm) were stained with hematoxylin and eosin, or with Kinyoun's acid-fast stain. Sections were also probed with monoclonal antibody for the

detection of the inducible nitric oxide synthase protein (Santa Cruz Biotechnology) by immunohistochemistry, as previously described (Cattoretti, 1992), and all were viewed on a Zeiss Axiophot, photographed utilizing a didymium filter and Lumiere 100 speed film (Kodak).

Transmission Electron Microscopy

Normal C57BL/6, BALB/C, and *Csfm^{op}/Csfm^{op}* mice and their controls were harvested, and their lung tissue was inflated with fixative as described elsewhere (Morin et al., 1994). Lungs were inflated with approximately 2 ml of a 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) buffered solution. Whole lungs were then excised, immersed in the fixative solution, and fixed overnight. Tissue was further dissected, postfixed in 1% osmium tetroxide, en bloc stained in uranyl acetate, dehydrated in a graded ethanol series, then embedded in LX112 (Ladd Research, Burlington, VT) in flat molds, or beem capsules, and polymerized at 60°C. Thick sections were cut and stained with toluidine blue for evaluation by light microscopy. For ultrastructural studies, thin sections were stained with uranyl acid and lead citrate prior to examination on a JEOL 1200EX or JEOL 100CX transmission electron microscope.

Scanning Electron Microscopy

Normal BALB/C and C57BL/6 lung tissue was harvested and fixed as per the TEM samples. After overnight fixation, lung tissue was dissected and the mucosal surface was exposed. Tissue was postfixed in 1% osmium tetroxide, in 0.1 M sodium cacodylate buffer, dehydrated in a graded ethanol series, critical point dried, affixed on a specimen stub, mucosal surface exposed, sputter coated with gold palladium, and viewed with a JEOL 6400 scanning electron microscope.

Lymph Node Removal

BALB/C mice were challenged with 1×10^7 BCG Pasteur intranasally. At 4 and 24 hr post infection mice were overdosed on sodium pentobarbital. Lungs were exposed, and lymph nodes were visualized with the aid of an Olympus SZ3060 stereomicroscope. Three lymph nodes were removed from the bronchotracheal nodes, and three nodes were removed from the bronchopulmonary and pulmonary nodes. Removed lymph nodes were immersed in petri dishes containing 6 ml of PBS twice, then homogenized in a dounce homogenizer in 500 μl of PBS supplemented with 0.05% Tween 80. The homogenate was then plated on 7H10 plates, incubated at 37°C for 3 weeks, and cfu were enumerated. Lung parenchymal tissue was excised as well, approximate in size to that of the lymph nodes, homogenized and plated as above, as a positive control. *Csfm^{op}/Csfm^{op}* mice and their homozygous wild-type controls were similarly infected with *M. tuberculosis* at a dose of 1×10^7, and sacrificed 36 hr post infection by overdose of sodium pentobarbital as above. Lymph nodes were visualized with the aid of an Olympus SZ3060 stereomicroscope placed within the biosafety cabinet. The microscope was equipped with a ccd camera, attached to a video monitor placed outside the biosafety cabinet, enabling microdissection of the *M. tuberculosis*-infected lymph nodes. Once excised, lymph nodes were placed in a petri dish containing PBS supplemented with 0.05% Tween 80, 100 μg/ml carbanicillin, and 100 μg/ml cycloheximide, homogenized in a dounce homogenizer, and plated as above.

Isolation of Lung Mucosal Tissue

Csfm^{op}/Csfm^{op} mice and C57BL/6 controls were infected IN with 1×10^7 *M. tuberculosis* as above ($n = 3$). Lungs and airway were removed from each mouse at 36 hr and 2 weeks post infection and placed in dishes containing PBS supplemented as above. Lungs were microdissected with the aid of the stereomicroscope, and mucosa was separated from lung parenchyma. As much parenchyma was stripped away as possible. All lungs were dissected prior to homogenization to ensure that the tissue taken was comparable between groups. Mucosa was homogenized in 500 μl supplemented PBS and plated on 7H10 agar plates. Colony-forming units (cfu) were enumerated after 3 weeks incubation at 37°C.

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